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TITLE: Human telomerase catalytic subunit variants

DATE-ISSUED: January 8, 2002

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
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US-CL-CURRENT: 435/194; 435/320.1, 435/325, 435/440, 435/455, 435/69.1, 435/70.1, 514/44, 530/350, 536/23.1, 536/23.5

CLAIMS:

What is claimed is:

1. A polynucleotide encoding a variant of human telomerase reverse transcriptase (hTRT), said variant having processive catalytic activity and comprising a deletion of at least 10 amino acids from region 192-323 or 415-450 of SEQ. ID NO:2.

2. The polynucleotide of claim 1, wherein the variant comprises a deletion of at least 25 amino acids from region 192-323 or 415-450 of SEQ. ID NO:2.

3. The polynucleotide of claim 1, further comprising a promoter sequence operably linked to the nucleotide sequence encoding the hTRT variant.

4. The polynucleotide of claim 1 that has a deletion of at least one region encoding exactly amino acids 192-323, 200-323, 200-271, 222-240, or 415-450 of SEQ. ID NO:2.

5. The polynucleotide of claim 1 that does not comprise a deletion in the region encoding amino acids 415-450.

6. The polynucleotide of claim 5, further comprising a promoter sequence operably linked to the nucleotide sequence encoding the hTRT variant.

7. A method for increasing the proliferative capacity of a human cell in vitro, comprising expressing the polynucleotide of claim 6 in the cell, thereby increasing its proliferative capacity.

8. A method for increasing the proliferative capacity of a human cell in vitro, comprising expressing the polynucleotide of claim 3 in the cell, thereby increasing its proliferative capacity.

9. A method for producing a variant telomerase reverse transcriptase, comprising expressing the polynucleotide of claim 1 in a host cell or in a cell-free expression system.

10. A cell comprising the polynucleotide of claim 1.

11. The cell of claim 10, that is a human cell.

1: Ann Plast Surg. 1990 Jan;24(1):17-23. Related Articles, Lin

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Effects of ischemia on ulcer wound healing: a new model in the rabbit ear.

Ahn ST, Mustoe TA.

Department of Plastic Surgery, Catholic University Medical College, Seoul, Korea.

To study the effects of alteration of blood flow on wound healing in rabbit ear ulcers, two models were designed that produced maximum ischemia and maximum congestion, respectively, with complete survival of the ear by selecti division of one or more of three arteries or veins and circumferential incisions. After selection of the best models from six variations, tissue perfusion was measured indirectly by venous oxygen tension, dermofluorometry, pulse oximet and skin temperature. Wound healing was measured seven days after creating 6-mm surgical ulcers. The tissue oxygen tension calculated from the venous oxygen tension fell to 30 mm Hg through days 1, 3, and 7 in the ischemic ears, and skin temperature and blood flow measured by dermofluorometry were similarly affected. However, there was no evidence of significant change in tiss perfusion except increased skin temperature in the congested ears. None of the ischemic wounds were epithelialized completely, but half of the control and congested wounds were. The granulation tissue formation was decreased (39% the area versus 63% of the area) and the infection rate was increased (20% versi 2%) significantly in the ischemic wounds compared with the control wounds bu not in the congested wounds. The ischemic ulcer model is reproducible and quantifiable and is potentially a useful model for examining agents to improve ulcer healing where blood flow is decreased. The congestion model showed no significant changes in blood flow or wound healing.

PMID: 2301878 [PubMed - indexed for MEDLINE]

1: Mol Med 1997 Jul;3(7):466-76 Related Articles, Links

Versatile retrovirus vector systems for regulated gene expression in vitro and in vivo.

Lindemann D, Patriquin E, Feng S, Mulligan RC.

Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA.

BACKGROUND: Several plasmid DNA-based mammalian expression systems have recently been developed which make it possible to manipulate gene expression via the administration of exogenous agents. In order to extend the application of these systems, we have developed retroviral vectors which allow for the controlled expression of inserted genes both in vitro and in vivo. MATERIALS AND METHODS: Two vector strategies which make use of the tetracycline-regulated gene expression system described by Gossen and Bujard were evaluated. In a first strategy, one virus was generated which encoded the tTA or rtTA transactivator gene product, and a second virus was generated in which expression of the gene of interest was dependent upon tetracycline-responsive transcriptional control elements placed either within the viral LTR or within the proviral transcriptional unit. In a second vector strategy, both components of the tet-regulatable system were incorporated into a single proviral genome in such a way that expression of both the transgene and the transactivator gene product were under control of tet-regulatable control elements. RESULTS: Both vector strategies resulted in the ability to regulate the expression of inserted genes. In one single virus configuration, gene expression could be regulated over 100X and the level of gene expression in the induced state was comparable to or greater than that achieved with standard LTR-based vectors. The use of different deletions in the viral LTR made it possible to generate a number of vectors which provide for a four-fold range of levels of expression of inserted genes in the induced state. Studies in mice with transduced cells demonstrated that gene expression could be induced in vivo by manipulation of tetracycline for at least 48 days. CONCLUSIONS: The availability of highly transmissible, regulatable retroviral vectors should greatly facilitate studies in which it is of interest to manipulate the expression of specific genes in vitro or in vivo.

PMID: 9260158 [PubMed - indexed for MEDLINE]

American Journal of Pathology, Vol. 154, No. 1, January 1999

Animal Model

Transforming Growth Factor-β1 Fails to Stimulate Wound Healing and Impairs Its Signal Transduction in an Aged Ischemic Ulcer Model

Importance of Oxygen and Age

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From the Departments of Surgery and Pathology† Northwestern University Medical School, Chicago, Illinois, and the Department of Biotechnology,‡ United States Surgical Corp., North Haven, Connecticut*

Clinical trials of exogenous growth factors in treating chronic wounds have been less successful than expected. One possible explanation is that most studies used animal models of acute wounds in young animals, whereas most chronic wounds occur in elderly patients with tissue ischemia. We described an animal model of age- and ischemia-impaired wound healing and analyzed the wound-healing response as well as the transforming growth factor (TGF)-β_1 effect in this model. Rabbits of increasing ages were made ischemic in the ear where dermal ulcers were created. Histological analysis showed that epithelium ingrowth and granulation tissue deposition were significantly impaired with increased age under ischemia. TGF-β1 stimulated wound repair under both ischemic and non-ischemic conditions in young animals, although it showed no statistical difference in aged animals. Procollagen mRNA expression decreased under ischemic conditions and with aging. Neither TGF-β1 nor procollagen α1(I) mRNA expression increased in response to TGF-β1 treatment under ischemia in aged animals. Therefore, the wound-healing process is impaired additively by aging and ischemia. The lack of a wound-healing response to TGF-β1 in aged ischemic wounds may play a role in the chronic wounds. *(Am J Pathol 1999, 154:301–309)*

Many growth factors tested in animal models appear to be promising therapeutic agents promoting wound healing.[1] However, clinical trials of growth factors on treating chronic wounds have been less encouraging.[2,3] We reasoned that preclinical studies using young animals may not be suitable for predicting growth factor effect on human chronic wounds, which are primarily a problem of aged patients who have local tissue hypoxia. Previously, most preclinical studies have been done on acute wounds in healthy young animals.[1,4] Impaired wound-healing models that have been used in many previous studies include young animals with the impaired conditions produced by injecting glucocorticoids, treating with radiation, or decreasing the blood supply to the wound.[5–9] Some preclinical studies have examined age effects on wound healing, but truly aged animals (defined as those at the age when one-half of the studied population has died due to natural causes) under the condition of tissue ischemia have not been studied.[5,10–12] However, most chronic wounds occur in aged patients with varying degrees of local tissue ischemia secondary to scarring, fibrin cuffing, edema and increased venous pressure in venous stasis, pressure in pressure ulcers, small artery disease, and edema in diabetic ulcers.[2,13–15] Therefore, the questions are whether some of the growth factors are less effective in promoting wound healing of aged ischemic animals and whether those altered effects are due to altered gene expression and signal transduction resulting from aging and ischemia.

In general, wounds heal more slowly in healthy elderly human and animals.[16–19] Studies suggest that aging is accompanied by altered inflammatory response,[20,21] decreased fibroblast proliferation,[22] delayed angiogenesis,[23] reduced deposition of specific extracellular matrix components,[24,25] and slower re-epithelialization.[18] How-

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ever, the effect of tissue ischemia, a common denominator of a number of other disease processes, such as stroke, myocardial infarction and ischemia reperfusion injuries that have a high incidence in the elderly population, has not been studied in aged systems. As the aged population grows, wound healing under impaired conditions secondary to ischemia will increase patient morbidity and mortality after surgery or tissue injury. Clinicians have observed that aged healthy patients can have surgery and heal with few complications.[26] However, aged patients with the additive conditions that contribute to local wound tissue ischemia tend to have a higher incidence of surgical wound dehiscence and are at higher risk for developing chronic wounds. Thus, we hypothesize that aging and ischemia have an additive effect on wound healing, and growth factor effects in promoting wound healing may be minimized under ischemic conditions.

Transforming growth factor (TGF)-β1, one of the strongest stimulators of wound healing as shown in preclinical studies,[1,5,27–29] has been associated with various stages of tissue repair.[27,29] TGF-β1 has been shown to stimulate wound healing in young, normal, and ischemic animal models[29] when applied locally. Systemic administration of TGF-β1 stimulated wound healing in a middle-aged rat incisional model.[5] A study of the reduced healing rate seen in aged human females suggested a possible role of reproductive hormones in wound healing, and analysis showed a significant difference in re-epithelialization with aging.[30] In profound wound healing, deficits of age and ischemia are unknown. Aged mouse dermal cells express less TGF-β1 mRNA *in vivo*.[31] It has also been shown in vitro that TGF-β1 binding affinity is impaired under hypoxic conditions in young dermal fibroblasts.[32] Our experimental data suggest that aging and ischemia may have additive effects on TGF-β1 mRNA expression. Collagen synthesis and deposition into the wound is essential during wound healing, and TGF-β1 is the strongest stimulator of collagen synthesis in wound healing.[27] Thus, it is very important to study collagen gene expression under different conditions during wound healing and after TGF-β1 treatment in both young and aged animals. This research will help evaluate the effects of aging and ischemia and the potential benefit of TGF-β1 treatment in wound healing. It will also help us learn about the signal transduction of growth factors and the potential effect of aging and ischemia on signal transduction at the molecular level.

Materials and Methods

Dermal Ulcer Wound-Healing Model in Aged Rabbits

New Zealand White male rabbits (Hazelton, Norwalk, CT), young adult (6 months, ranging from 5 to 7 months), retired breeder rabbits, middle-aged (30 months, ranging from 29 to 31 months), upper middle-aged (36 months, ranging from 35 to 37 months), and aged (60 months, ranging from 58 to 62.5 months) were acclimated and kept under standard conditions in the Northwestern University Animal Care Center. As the lifespan of retired breeder laboratory rabbits is ~5 years, which is shorter than that of normal laboratory rabbits, this age is comparable to that of humans in their 7th to 8th decades. This study and its surgical procedures have been approved by the Northwestern University Animal Care and Use Committee. The surgical procedures were performed as previously described[9,33] after anesthetizing the rabbits with Ketamine (60 mg/kg) and Xylazine (5 mg/kg). Briefly, one of the rabbit ears was made ischemic by dissecting the rostral and central arteries and interrupting the entire dermal circulation, preserving only the major three veins and the smallest caudal artery. Three full-thickness, 6-mm-diameter circular wounds were created extending down to bare cartilage. The contralateral ear vessels were left undisturbed and served as matched non-ischemic controls. All of the wounds were covered with an occlusive polyurethane dressing (Tegaderm, 3M, Minneapolis, MN) for 12 days. The wounds were bisected and analyzed histologically based on a previous study that showed these wounds were minimally contracted.[29] Re-epithelialization rate, percentage of full re-epithelialization, and new granulation tissue formation in all matched wounds were measured as previously described.[29,33]

TGF-β1 Effects in Aged Dermal Ulcers

Recombinant human (rh)TGF-β1 (1 μg/wound; Amgen, Thousand Oaks, CA) was topically applied once to the wounds immediately after wounding. TGF-β1 was also topically applied to the ischemic wounds made in young, middle-aged, and aged rabbit ears. The dose of TGF-β1 (1 μg/wound) was chosen based on previous studies that demonstrated it was the optimal dose for ischemic wound healing.[34] In all rabbits, whether young or aged, the wounds on contralateral ears served as a paired control and were treated with vehicle alone (PBS). The growth factors were purified to homogeneity by conventional techniques and assayed for endotoxin before use. This growth factor has been tested *in vitro* and *in vivo*, and no difference in biological effects was found between recombinant and natural growth factor derived from macrophage.[35] The rabbit ear ulcers were harvested and evaluated histologically at day 12 after wounding as previously described.[5,29,34]

Statistical Analysis

All of the wounds were created and harvested in a matched fashion, and the data were collected in the same manner allowing paired analyses with each animal serving as its own control. A paired two-tailed Student's *t*-test (Epistat program, Epistat Service, Richardson, TX) was used to detect differences between non-ischemic and ischemic wounds in each age group and between TGF-β1-treated wounds and matched control wounds. Analysis of one-way variance was used to analyze the differences among different age groups in model development and TGF-β1-treated wounds. The χ^2 test was

Table 1. Primer Sequences and PCR Cycle Information

	GAPDH	TGF-β1	Procollagen 1(I)
Upper Primer	5′-CCA TGT TCG TCA TGG GTG TGA ACC A-3′	5′-CTT CAG CTC CAC AGA GAA GAA CTG C-3′	5′-TTC AGC TTT GTG GAC CTC CGG CTC
Lower Primer	5′-CAT GAG TCC TTC CAC GAT ACC AAA G-3′	5′-CAC GAT CAT GTT GGA CAA CTG CTC C-3′	5′-CTG AGG GAT GCC ATC TCG GCC-3′
Cycling (25 cycles each)	94 °C for 45 seconds, 55 °C for 45 seconds, 72 °C for 90 seconds	94 °C for 45 seconds, 55 °C for 45 seconds, 72 °C for 90 seconds	94 °C for 45 seconds, 50 °C for 45 seconds, 72 °C for 90 seconds

used to analyze the differences in the percentage of full re-epithelialization. Multivariate analysis (Epistat program) was used to analyze the relative responsiveness of adult *versus* young or aged animals.

Competitive Reverse Transcription Polymerase Chain Reaction

The wound granulation tissue in each rabbit was harvested as previously described.[36,37] All three wounds from each ear were processed, and the total cellular RNA was extracted with guanidine thiocyanate/phenol-based reagent according to the manufacturer's instructions (TRI Reagent, Cincinnati, OH).[38] All reverse transcription (RT) reactions were performed simultaneously using a master mix to eliminate variability of RT efficiency. A total of 5.0 μg of each RNA sample, with acceptable purity (A260/A280 ratio ≥1.8) was converted to cDNA using Moloney murine leukemia virus reverse transcriptase and random primers (Gibco BRL, Grand Island, NY).

Specific polymerase chain reaction (PCR) primers for rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TGF-β1, and procollagen 1(I) were designed using conserved sequences from published Genbank complete and partial mRNA sequences of various species. Sequence information of all PCR primers used and the reaction conditions are listed in Table 1. The PCR products were confirmed by subcloning each into TA cloning vectors (TA cloning kit, Invitrogen, San Diego, CA) and sequencing analysis.[39,40] Nonhomologous competitive PCR fragments (mimic) were used as an internal standard to measure the desired product.[41,42] Mimic was created using a commercial kit (Clontech, Palo Alto, CA). Serial dilution of a known quantity of mimic was coamplified with a constant amount of the cDNA sample, which resulted in varying band intensities depending on the ratio between the mimic and the cDNA of interest. The reaction products were electrophoresed on 2% agarose gels. Gel photographs were quantified by densitometry imaging (Imaging Densitometer GS-670, Bio Rad, Richmond, CA), and the ratio of gene product to mimic was plotted against the known quantity of the mimic. At a ratio of one, each curve gives the corresponding concentration of gene product cDNA. Competitive PCR reactions designed to compare the experimental condition with the control were run simultaneously to allow relative comparison of the extrapolated ratios. Results were confirmed by repeating experiments within the same RNA extraction and with multiple rabbits.

Results

Histology studies showed depressed wound healing by age and ischemia. Young non-ischemic wounds had the most healing (Figure 1A), and young ischemic and aged non-ischemic wounds healed to a lesser degree (Figure 1, B and C). Aged ischemic wounds had severely impaired healing (Figure 1D), showing essentially no healing on day 12. Quantitative analysis of new granulation tissue deposition showed a moderate and progressive decrease with aging under non-ischemic conditions and a sharp decrease under ischemic conditions in both middle-aged and aged animals (Figure 2). Multivariate analysis showed that under non-ischemic condition, significant decrease of wound granulation tissue formation was found between age 30- and 6-month-old rabbits (41.7% decrease, $P < 0.01$) and 60- and 30-month-old rabbits (70.0% decrease, $P < 0.05$). Under ischemic condition, the decrease was 92.9% ($P < 0.01$) between 6- and 30-month-old rabbits, and no significant change was detected between 60- and 30-month-old rabbits. Young ischemic wounds showed a 65% decrease in new granulation tissue formation compared with their age-matched non-ischemic controls. Middle-aged and aged rabbits each had a more than 95% decrease compared with their age-matched control wounds (Figure 2). Histological analysis of hematoxylin and eosin (H&E)-stained wound tissue sections revealed a significant reduction of re-epithelialization by age and ischemia ($P < 0.01$) (Figure 3) as well. Multivariate analysis showed significant difference in re-epithelialization with aging: in non-ischemic wounds, 57.1% ($P < 0.01$) decrease between 30- and 6-month-old rabbits and 76.7% ($P < 0.01$) decrease between 30- and 60-month-old rabbits. In ischemic wounds, an 80% decrease ($P < 0.01$) was found between 30- and 6-month-old rabbits, and a 100% ($P < 0.05$) decrease was found between 30- and 60-month-old rabbits.

TGF-β1 has been found to stimulate wound healing in several models of young animal, including ischemic, diabetic, and radiation-impaired healing.[1] In this study, we found that TGF-β1 stimulated partial healing of non-ischemic wounds of aged animals at day 12 (Figure 4C), at which time wounds of young animals showed complete epithelialization (Figure 4A). Under ischemic conditions, TGF-β1 stimulated wound healing of young rabbits (Figure 4B), but it failed to promote the healing of aged rabbits (Figure 4D). Quantitative analysis of the H&E-stained wound section showed that TGF-β1 stimulated

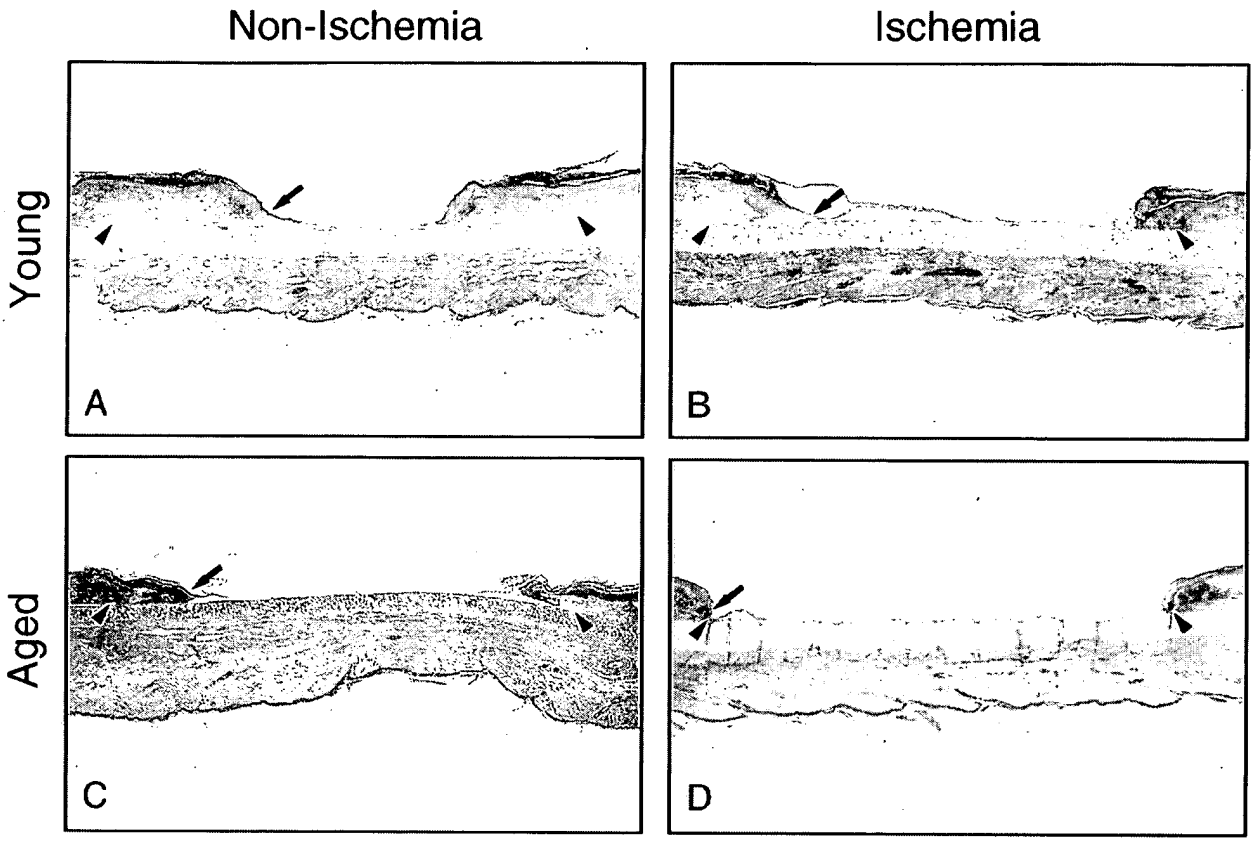

Figure 1. Day 12 wounds stained by H&E. Full-thickness wounds were created on ischemic (B and D) and non-ischemic (A and C) rabbit ears using a 6-mm biopsy punch. Severe delay of both granulation tissue formation and re-epithelialization were observed in aged (60 months) ischemic wounds (D) compared with aged non-ischemic wounds (C), young (6 months) ischemic (B), and young non-ischemic (A) wounds. Within each age group, ischemia caused delayed wound healing when A *versus* B and C *versus* D were compared. The migrating front of epithelial sheet is indicated by arrows, and the initial cutting sites are indicated by arrowheads. Magnification, ×40.

the growth of new granulation tissue to 136% in young animals and 100% in middle-aged animals, compared with the vehicle-treated control (Figure 5). Epithelialization was increased to 100% in young animals and 200% in middle-aged animals, compared with the vehicle-treated control (Figure 6). In either case, TGF-β1 failed to promote wound healing in aged rabbits (Figures 5 and 6). This is the first animal model showing that an impaired healing wound failed to respond to TGF-β1.

Expression of TGF-β1 mRNA was measured by competitive PCR analysis. In young non-ischemic wounds, TGF-β1 mRNA reaches its peak at approximately day 7.

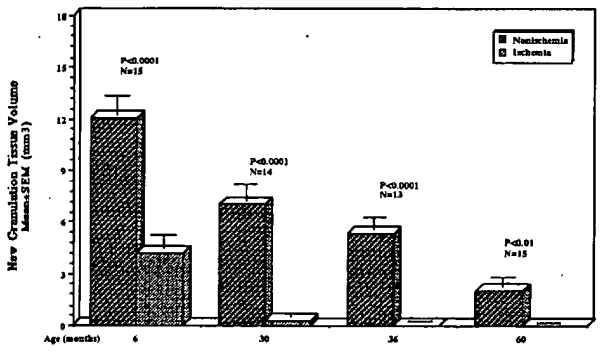

Figure 2. Effect of aging and ischemia on new granulation tissue formation. Day 12 wounds were harvested from both ischemic and non-ischemic animals of increasing age and stained with H&E, followed by quantification of granulation tissue formation using a calibrated lens reticule under a light microscope (×10). Reduction of granulation tissue formation was observed with increasing donor age and ischemia. The paired Student's *t*-test was used for statistical analysis.

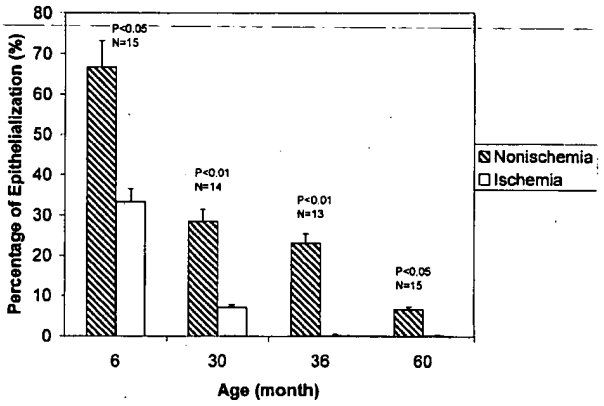

Figure 3. Effect of aging and ischemia on re-epithelialization. Wounds were harvested on day 12 after wounding from both ischemic and non-ischemic rabbit ears. Re-epithelialization was quantified histologically on H&E-stained tissue sections. Significant decreases in the percentage of complete re-epithelialization among different age groups and under ischemia *versus* non-ischemia were observed. The paired Student's *t*-test was used for statistical analysis.

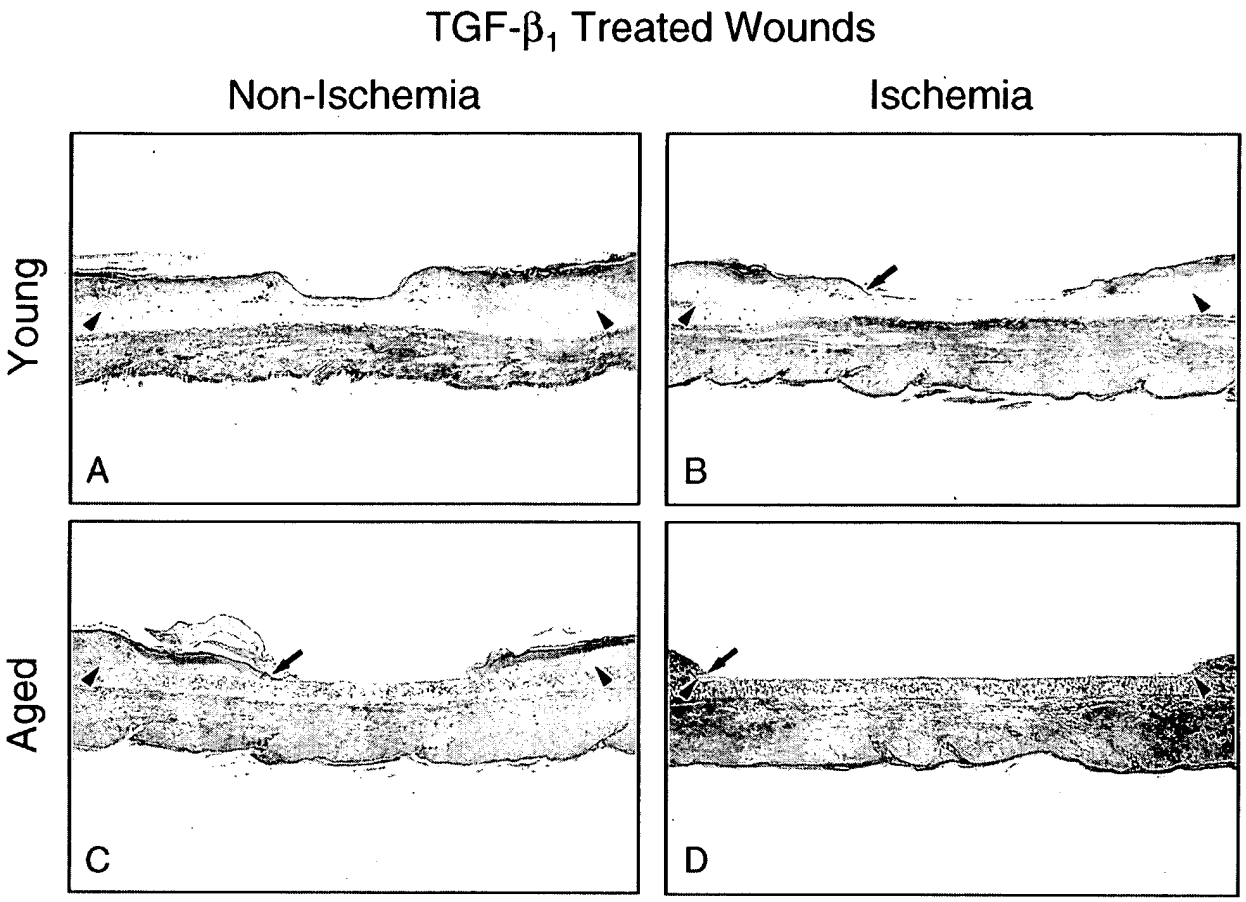

Figure 4. H&E staining of day 12 TGF-β1-treated wound (original magnification, ×40). Wounds were treated with TGF-β1 (1 μg/wound) at the time of wounding. Aged ischemic wounds (D) showed the least healing response to TGF-β1 treatment, as compared with young non-ischemic (A), young ischemic (B), and aged non-ischemic (C) wounds. Note that the two epithelial sheets have confronted each other in TGF-β1-treated young non-ischemic wounds (A). Within the same age group, the TGF-β1 effect is impaired by ischemia, when A *versus* B and C *versus* D were compared. The migrating front of epithelial sheet is indicated by arrows, and the initial cutting sites are indicated by arrowheads. Magnification, ×40.

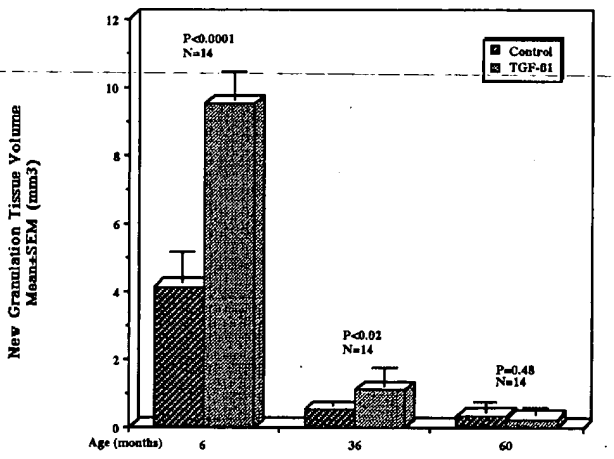

Figure 5. TGF-β1 fails to stimulate new granulation tissue formation of ischemic wounds from aged animal donors. Ischemic wounds were treated with TGF-β1 (1 μg/wound) at the time of wounding. Wounds were harvested on day 12 after wounding and evaluated histologically. PBS vehicle-treated wounds were used as the control for each age group. The aged animals (60 months) showed no increase in new granulation tissue formation, in contrast to a 138% increase in young animals (6 months).

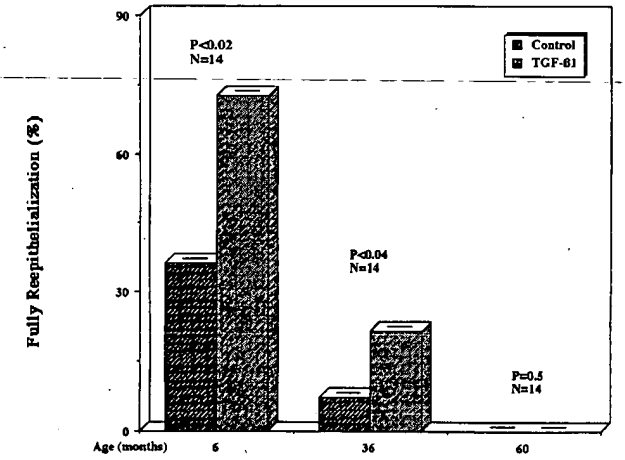

Figure 6. TGF-β1 fails to promote re-epithelialization of ischemic wounds from aged animal donors. Ischemic wounds were treated with TGF-β1 (1 μg/wound) at the time of wounding. Wounds were harvested on day 12 after wounding and evaluated histologically. PBS vehicle-treated wounds were used as controls for each age group. The aged animals (60 months) showed no increase in re-epithelialization in contrast to a 100% increase in young animals (6 months).

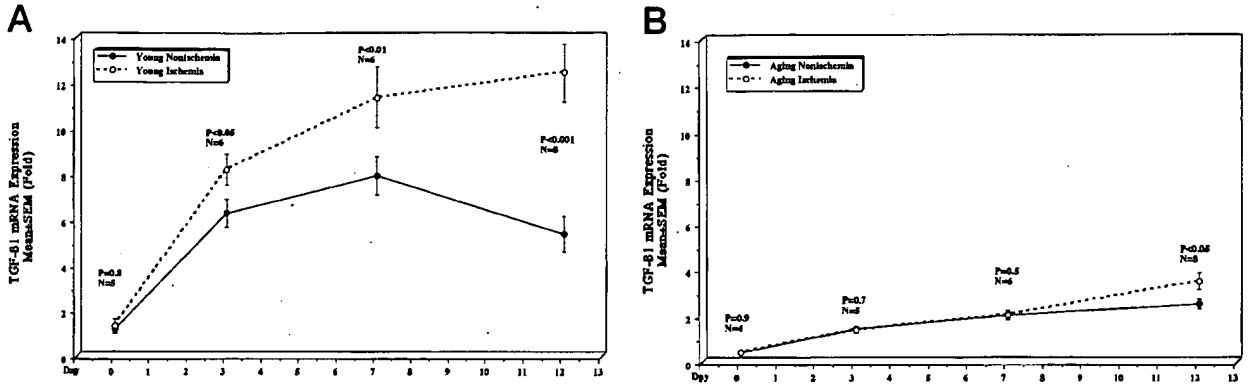

Figure 7. Ischemic effect on endogenous TGF-β1 mRNA expression. Ischemic wounds of both young and aged animals were harvested at days 3, 7, and 12 after wounding, using age-matched non-ischemic wounds as controls. Total RNAs were extracted, and the level of TGF-β1 mRNA with the progression of wound healing was quantified by competitive PCR analysis. Ischemia stimulated TGF-β1 mRNA expression in young (6 months) animal wounds (A). However, no obvious stimulation was observed in aged (60 months) animal wounds (B).

A significant up-regulation was also observed in ischemic wounds (Figure 7A). Aged rabbits showed a major decline in the level of TGF-β1 mRNA under both ischemic and non-ischemic conditions compared with the young rabbits. No significant difference was observed between ischemic and non-ischemic wounds of aged rabbits (Figure 7B). These data indicated that the aged rabbit wounds not only expressed lower levels of TGF-β1 mRNA but also lost responsiveness to ischemia in up-regulating TGF-β1 expression as young animals do. The level of type I procollagen was also quantified by competitive PCR analysis. In non-ischemic wounds, young rabbits expressed a peak level of type I collagen at approximately day 7 (Figure 8A), whereas in aged animals the expression was delayed but was still rising at day 12 (Figure 8B). Interestingly, ischemia down-regulated procollagen 1(I) expression in both young and aged rabbits (Figure 8, A and B). As an internal control, GAPDH showed no significant change of expression with aging and ischemia (unpublished observation).

When the wounds were treated with TGF-β1 growth factor, a consistent pattern of muted response to TGF-β1 treatment was demonstrated in aged animals. Aged ischemic wounds had a minimal response to TGF-β1 treatment in terms of TGF-β1 mRNA expression (Figure 9A) and procollagen expression (Figure 9B). As both TGF-β1 and collagen synthesis are endpoints of TGF-β1 stimuli, the data suggested that signal transduction in aged ischemic wounds was severely impaired due to age and low tissue oxygen supply.

Discussion

The rabbit ear wound-healing model was developed and used in our laboratory to study the effect of growth factors in wound healing under normal and ischemic conditions.[29,34,43] The model allows us to precisely measure the new granulation tissue formation and re-epithelialization in a matched control manner, because the ear cartilage splints the wound with minimal contraction.[29] This model is also ideal for harvesting the new granulation tissue for biochemical analysis of factors intrinsic to wound repair.[44] The pathogenesis of chronic wounds found in elderly patients (principally venous ulcers, diabetic ulcers, and pressure sores) is multifactorial, but a common factor in most is varying degrees of local wound tissue hypoxia. Previous animal models have failed to

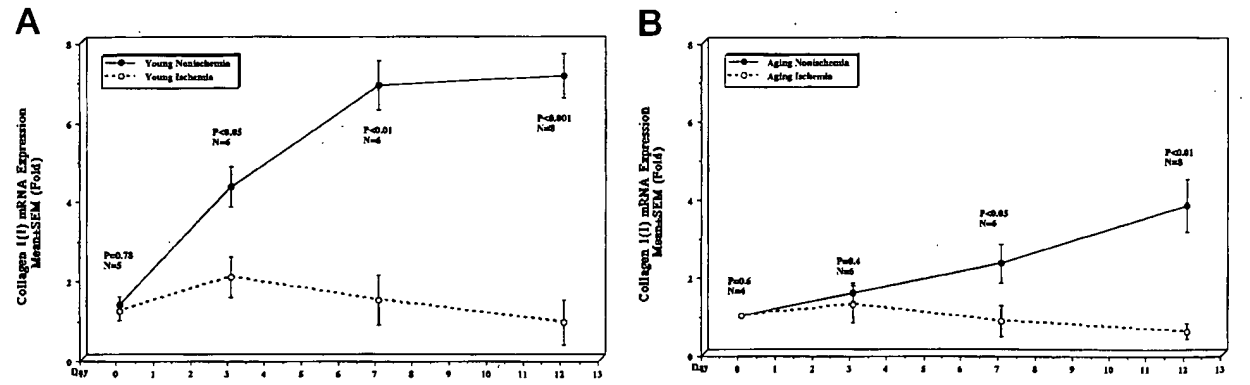

Figure 8. Ischemic effect on collagen-α1(I) mRNA expression. Excisional wounds of 6-mm were generated and left to heal for 12 days before harvest. Total RNAs were extracted for reverse transcription and PCR amplification. Level of collagen-α1(I) mRNA with the progression of wound healing was quantified by competitive PCR analysis. The level of collagen-α1(I) mRNA expression decreased in both ischemic wounds from young (6 months, A), and aged (60 months, B) animal donors.

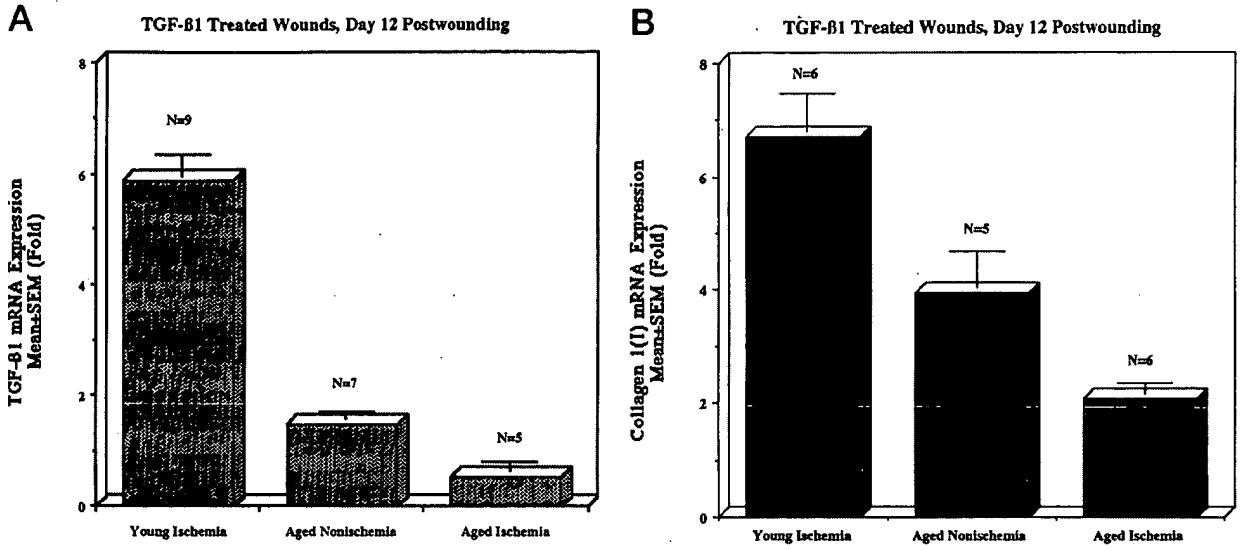

Figure 9. Expression of TGF-β1 and collagen-α1(I) mRNA in TGF-β1-treated wounds. Wounds were treated with TGF-β1 growth factor (1 μg/wound) immediately after wounding. Wounds were harvested at day 12 after wounding, and RNAs were extracted for reverse transcription and PCR. Level of TGF-β1 and collagen 1(I) mRNA expression were quantified by competitive PCR under aging and ischemic conditions. Both factors of age and ischemia repressed expression of TGF-β1 mRNA (A) and collagen-α1(I) mRNA (B) in TGF-β1-treated wounds.

consider the possible additive effect of aging and ischemia and thus have significant limitations in fostering understanding of human chronic wounds. In this study, we developed an aged ischemic model using 60-month-old rabbits, which is equivalent to human life of the 7th to 8th decades.[37] We observed profound impairment of granulation tissue formation and re-epithelialization under ischemic conditions with aging. Compared with the young non-ischemic model in which complete healing takes approximately 12 to 14 days, the healing in our aged ischemic model takes more than 20 days. Additionally, we have shown that TGF-β1 failed to promote wound healing in aged ischemic wounds. This is the first time that a wound-healing animal model showed no response to TGF-β1 treatment. It may explain some of the difficulties of clinical trials of growth factor on human chronic wounds. It is likely that our model will provide a better opportunity for the study of underlying biochemical and pathological mechanisms in chronic wound healing and allow re-evaluation of the vulnerary effect of growth factors under the specified conditions.

Tissue ischemia is known to be one of the most significant factors leading to chronic wounds.[45] To examine the effect of tissue oxygenation, we have carefully studied the possible reversal of the wound-healing deficit in ischemic wounds by hyperbaric oxygen.[34] It has shown that treatment of ischemic wounds with hyperbaric oxygen partially reversed the wound-healing deficit in both granulation tissue formation and epithelialization compared with the nontreated ischemic control.[34] More recent studies indicated that, in addition to its metabolic effects, low oxygen tension induces gene expression of several growth factor genes that are important in promoting wound healing, such as platelet-derived growth factor (PDGF)-BB, vascular endothelial growth factor (VEGF), and TGF-β1 as is shown in this report and by others.[46–48] It was also reported that hypoxia decreases TGF-β1 receptor binding and synthesis in dermal fibroblasts.[32] Presumably, heme-based proteins function as oxygen sensors leading to a series of chemical steps. Increasing evidence suggests that the chemical responses generated from oxygen sensing have signal-transducing effects.[49] The identification of hypoxia-inducible factor (HIF) provided a molecular basis for oxygen-induced gene regulation, the expression of which is regulated by cellular oxygen tension.[50,51]

Aging itself has an overt effect on gene expression[55,56] as well as on many aspects of biochemistry that are pertinent to wound healing. *In vitro* studies with cell culture showed an age-related decline of epidermal growth factor receptor-binding affinity, phosphorylation, and internalization[52,53] as well as activation of mitogen-activated protein kinase.[54] An *in vivo* study demonstrated that aged ischemic wounds have a dramatic decrease in PDGF receptors.[37] In this study, we found that aged animals responded very differently to added stress compared with young animals. Hypoxia up-regulated the level of TGF-β1 mRNA in wounds of young animals, although it elicited only a mild response in aged animals. *In vitro*, we found that aged keratinocytes migrate more slowly in response to hypoxia, whereas young keratinocytes migrate faster under the same condition (Y.-P. Xia, Y. Zhao, A. Chen, R. Galliano, and T. A. Mustoe, manuscript in preparation). It is likely that the regulatory machinery or the signaling pathway involved in stress management deteriorates with age, and therefore the ability of aged cells to survive environmental stress is reduced.

As TGF-β1 has a broad effect on all phases of wound repair, we examined the TGF-β1 effect on wound repair in our aged ischemic model. Previous studies have shown that in the inflammatory phase of repair, release of TGF-β1 from platelets increases chemotaxis of inflammatory cells into the wound site.[57,58] During the inflammation and after it has subsided, TGF-β1 induces both angio-

genesis[59] and extracellular matrix accumulation,[60] which continues through the remodeling phase of repair. Extracellular matrix production results from the effects of TGF-β1 on fibroblast, which include chemotaxis, proliferation, and induction of the synthesis and release of the matrix proteins. Additionally, TGF-β1 has an autoactivation function[61]; therefore, binding to its own receptor could stimulate expression of an elevated level of TGF-β1. Based on these facts, we analyzed the effect of TGF-β1 on granulation tissue formation, epithelialization, collagen synthesis, and induction of endogenous TGF-β1 mRNA expression in our animal model. We found that although TGF-β1 has been shown to stimulate wound healing in several other animal models under both normal and impaired conditions,[61] it failed to stimulate wound healing in the aged-ischemic rabbit model as described in this paper. Given the failure of wounds to respond to TGF-β1 and the depressed levels of downstream signal transduction events in response to TGF-β1 (autoinduction of TGF-β1 and collagen synthesis), it is evident that signal transduction for TGF-β1 is impaired under aged-ischemic conditions, which could be due to depressed TGF-β1 receptor level, altered kinase activity, or other downstream signaling events.

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